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Next-generation capillary electrophoresis–mass spectrometry approaches in metabolomics

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Capillary electrophoresis–mass spectrometry has shown considerable potential for profiling polar ionogenic compounds in metabolomics. Hyphenation of capillary electrophoresis to mass spectrometry is generally performed via a sheath–liquid interface. However, the electrophoretic effluent is significantly diluted in this configuration thereby limiting the utility of this method for highly sensitive metabolomics studies. Moreover, in this set-up the intrinsically low-flow property of capillary electrophoresis is not effectively utilized in combination with electrospray ionization. Here, advancements that significantly improved the performance of capillary electrophoresis–mass spectrometry are considered, with a special emphasis on the sheathless porous tip interface. Attention is also devoted to various technical aspects that still need to be addressed to make capillary electrophoresis–mass spectrometry a robust approach for probing the polar metabolome.

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Introduction

The major and ultimate aim of metabolomics is to obtain an answer to a specific biological or clinical question [1]. For this purpose, advanced analytical separation techniques are generally used for the global profiling of endogenous metabolites in biological samples [2••]. Currently, the profiling of endogenous metabolites is commonly performed with mass spectrometry (MS) in combination with an on-line front-end chromatographic separation method [3,4]. Despite significant developments in liquid chromatography column technology and methodology, such as hydrophilic interaction liquid chromatography, the selective and efficient analysis of highly polar and charged metabolites is still highly challenging. Capillary zone electrophoresis, referred to here as CE instead of CZE, separates compounds on the basis of differences in their intrinsic electrophoretic mobility, which is dependent on the charge and size of the analyte, in a capillary filled with separation buffer only under the influence of an electric field. Therefore, CE is highly suited for the analysis of polar ionogenic metabolites. Moreover, as the separation mechanism of CE is fundamentally different from chromatographic-based separation techniques, a complementary view on the composition of metabolites present in a given biological sample is provided. In comparison to chromatographic-based methods the separation efficiency of CE is very high as there is no mass transfer between phases. Actually, under ideal conditions the only source of band broadening in CE is from longitudinal diffusion.

A critical need for metabolomics is also the introduction of analytical methods allowing metabolic profiling of those samples for which the amount is severely limited [5]. CE–MS can be considered an attractive microscale analytical platform for this purpose, as in CE nanoliter injection volumes are employed from (sub-)microliter sample amounts. Therefore, CE–MS is highly suited for the analysis of polar ionogenic metabolites in ultra-small biological samples, as has been recently demonstrated for the analysis of cerebrospinal fluid of mice and extracts from small tissues or a single cell [6–8].

At present, the use of CE–MS for metabolomics studies is disproportionately low in comparison to other analytical separation techniques [2••]. The scientific community still perceives CE–MS as a technically challenging approach suffering from a relatively poor reproducibility and sensitivity [9]. An important reason for this perception is lack of expertise with this technology. In this context, it is of interest to note that CE–MS has been used for the global profiling of native peptides and endogenous metabolites in a clinical context for more than a decade now [10•,11–13]. For example, Mischak and co-workers have analyzed peptides in more than 20 000 human urine samples at different laboratories with an acceptable inter-laboratory reproducibility [10•,14].

Over the past few years, various novel CE–MS approaches have been developed which show a strong potential for improving the sensitivity/metabolic coverage and sample throughput in metabolomics. In this paper, attention will be paid to advancements that significantly improved the analytical performance, particularly with regard to improving the metabolic coverage, of CE–MS for metabolomics studies. Analytical aspects that still need to be addressed

to make CE–MS a viable approach in the metabolomics field are also discussed. Strategies to improve the stability of CE–MS in terms of migration times, data pre-processing aspects, procedures for the identification of metabolites and preconcentration techniques to improve the loadability of CE are not covered in this paper. The reader is referred to more dedicated literature for an overview concerning these topics [15–19,20•,21].

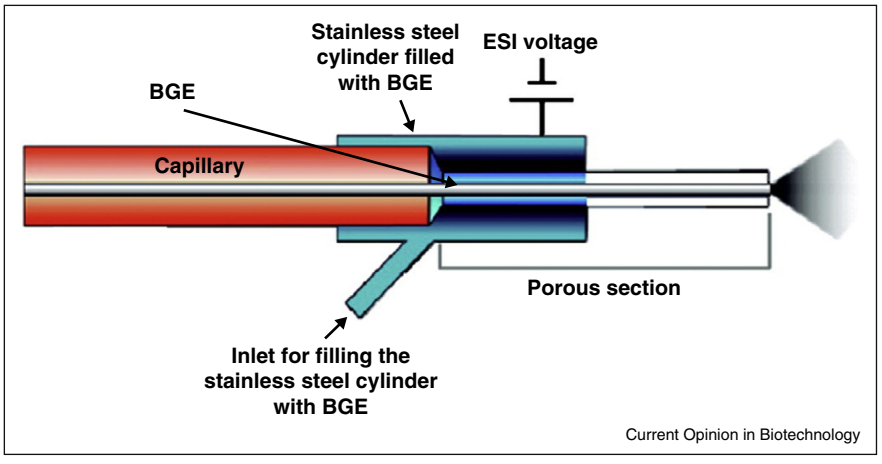
Interfacing techniques for seamless integration of CE and ESI–MS

CE is fundamentally a low flow nanoscale separation technique reaching its optimal separation performance at very low flow-rates, which is typically in the range of 20–100 nL/min depending on the pH of the separation buffer when using a bare fused-silica capillary. Actually, a high separation resolution is obtained in CE by solely separating the compounds on the basis of their electrophoretic mobilities, that is, under (near-)zero electro-osmotic flow conditions. The intrinsically low flow-rates of CE are also advantageous from a viewpoint of the ESI mechanism. In ESI, smaller droplets are generated under low-flow separation conditions, which results in a more efficient desolvation and an improved transfer of ions to the MS [22–24]. Moreover, at very low flow-rates ($\leq$20 nL/min) ion suppression is significantly reduced resulting in an improved concentration sensitivity [22], which is important for in-depth metabolic profiling studies.

In a standard CE set-up both ends of the separation capillary are immersed in buffer vials to which electrodes are added to provide a high voltage gradient. To couple CE to MS, the outlet vial must be replaced by an interface to close the electrical circuit and which provides contact with the ESI stream. Therefore, a CE–MS interface needs to apply voltage to the capillary outlet while maintaining independent CE and ESI electrical circuits. A co-axial solvent delivery as a terminal electrolyte reservoir (i.e., a sheath-liquid interface) and various other interfacing techniques have been subsequently developed to enable the hyphenation of CE to MS. So far, most CE–MS-based metabolomics studies have been performed with a sheath–liquid interface [13,25–30]. CE–MS approaches utilizing a sheath–liquid interface for global metabolic profiling studies were first developed by Soga and co-workers [12,31]. The sheath–liquid interface, originally developed by Smith and co-workers [32], has been used for a broad range of bio-analytical applications with acceptable analytical figures of merit. However, the sheath–liquid is generally provided at a flow-rate between 5 and 10 μL/min, thereby significantly diluting the CE effluent resulting in compromised detection sensitivities for metabolomics applications. Still, an important advantage of the sheath–liquid interface is that the composition of the sheath–liquid can be tuned to modify the ionization efficiency without affecting CE selectivity and efficiency. For example, to improve the detection sensitivity, supplementation of the sheath–liquid with modifiers has been investigated [33]. Enhanced supercharging of analytes in ESI–MS has also been explored by adding various supercharging agents to the sheath–liquid [34]. The effect of these agents on metabolic profiling studies by CE–MS needs to be studied. Overall, considering the fact that both CE and ESI–MS perform most optimally at low flow-rate conditions, the coupling of CE to MS should preferably be carried out via an interfacing technique which effectively utilizes the inherently low flow separation property of CE and the improved ESI efficiency under these conditions.

Currently, the design of new interfacing techniques for CE–MS and assessing their potential for proteomics and metabolomics studies is an active area of research [35–41]. New methods that abolish or minimize the usage of a

Figure 1



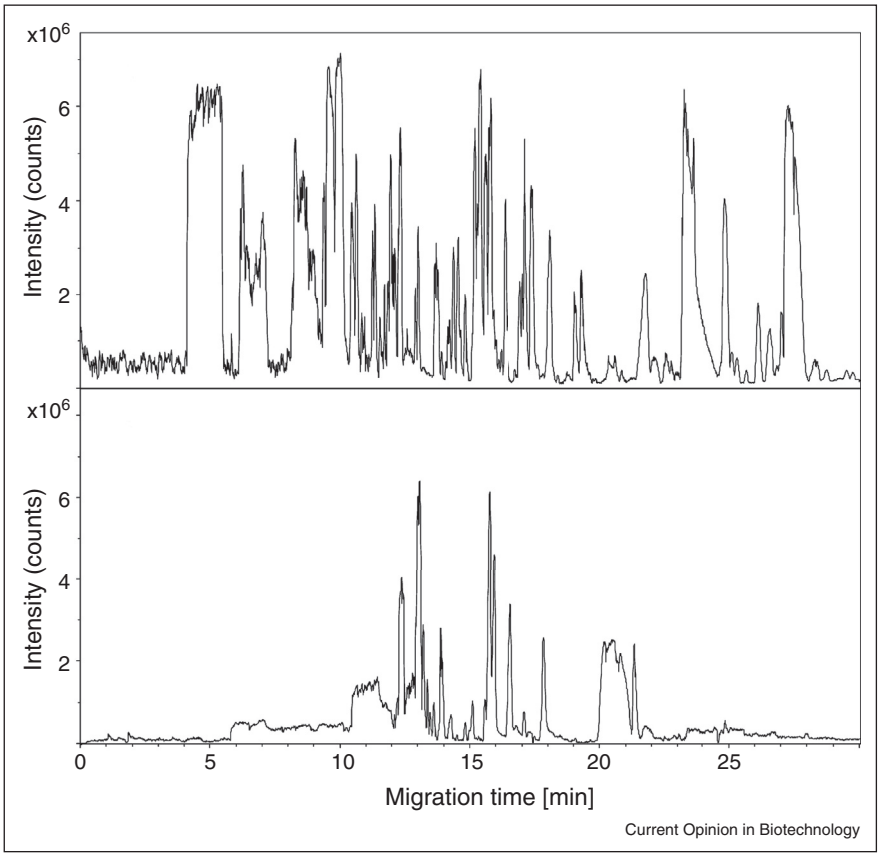
Design of the sheathless porous tip interface. A scheme of the porous tip interface, originally developed by Moini, is depicted. *Source*: Reproduced from [43] with permission.

sheath–liquid have been introduced to the market or are on the verge of being commercialized. In this context, three interfacing designs have been evaluated in detail for a wide range of bio-analytical studies, that is, the flow-through microvial interface, the sheathless porous tip interface and the electrokinetic-based sheath–liquid interface [42–44]. Concerning metabolomics, thus far the most promising results have been obtained by the sheathless porous tip interface (Figure 1), which was developed by Moini [43]. In this design, the porous tip interface was created by removing the polyimide coating of the capillary outlet and etching the capillary wall with 49% solution of hydrofluoric acid to a thickness of about 5 μm. The etched conductor was inserted into an ESI needle which was filled with separation buffer. Redox reactions of water at the ESI needle and transport of these small ions through the porous tip into the capillary provides the electrical connection for the ESI and for the CE outlet electrode. The sheathless porous tip design is especially useful for interfacing narrow (<30 μm i.d.) capillaries and for low flow-rate (<20–30 nL/min) nano-ESI–MS analyses [45••].

Enhancing the coverage of the polar metabolome

The performance of CE–MS utilizing a sheathless porous tip interface has been evaluated for the profiling of cationic metabolites in human urine (1:1 dilution with separation buffer) at low-pH separation conditions resulting in an information-rich metabolic profile [46]. The use of the sheathless interface enhanced the concentration sensitivity by over two orders of magnitude while maintaining high separation efficiency relative to the use of a sheath–liquid interface. This approach allowed for an improved coverage of the urinary metabolome with nanomolar detection limits for a broad range of polar ionogenic metabolites (Figure 2). Approximately 900 molecular features were detected with sheathless CE–MS, whereas 300 were found with sheath–liquid CE–MS. The enhanced sensitivity resulted in the detection of many compounds, including many low abundance ions above *m/z* 300, such as small peptides. Hirayama and co-workers also assessed the performance of this approach for urinary metabolomics and found a tenfold increase in the number

Figure 2



Comparison of sheathless and sheath–liquid CE–MS for metabolic profiling of human urine. (Top) Base peak electropherogram (*m/z* 50–450) of human urine obtained with sheathless CE-MS using a porous tip sprayer. Conditions: separation buffer, 10% acetic acid (pH 2.2); sample injection, 2.0 psi for 30 s (1% of capillary volume). (Bottom) Base peak electropherogram (*m/z* 50–450) of human urine obtained with CE–MS using a sheath–liquid interface. Conditions: separation buffer, 10% acetic acid (pH 2.2); sample injection, 0.5 psi for 30 s (1% of capillary volume). *Source*: Reproduced from [46] with permission.

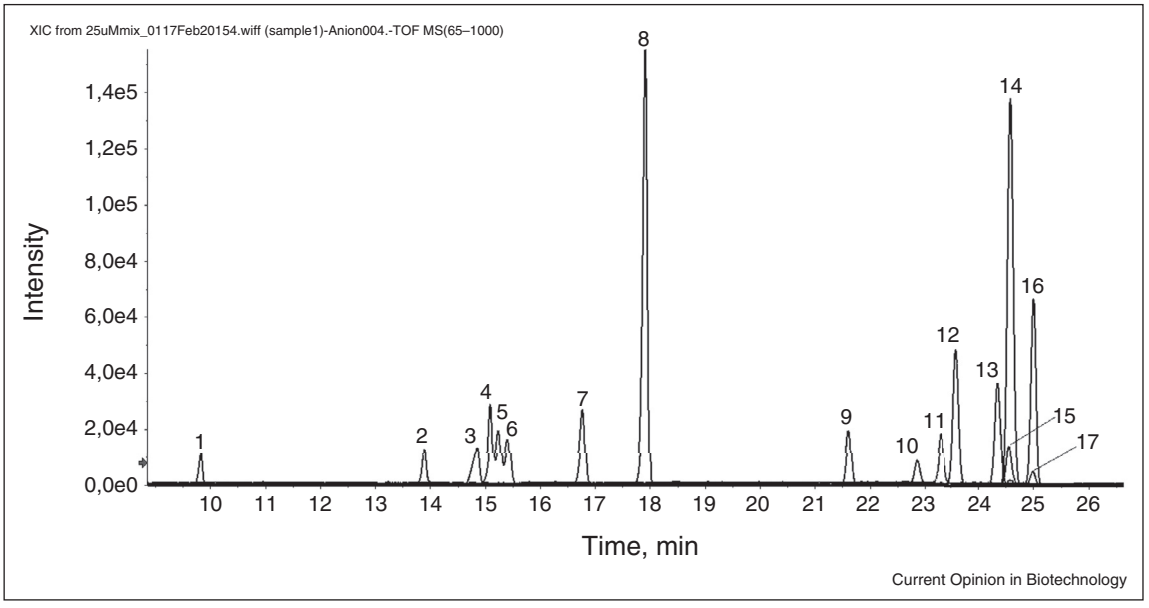
of detected peaks compared with conventional CE–MS methods [47•]. Though a single sheathless porous tip capillary could be used for more than 180 successive runs of a tenfold-diluted human urine sample, the long-term performance of this new CE–MS interface still needs to be assessed in more extended studies analyzing large numbers of diverse clinical samples.

Sheath–liquid CE–MS approaches for anionic metabolic profiling used in reversed polarity CE mode lack robustness due to oxidation and corrosion of the stainless steel ESI spray needle under these conditions, unless a platinum ESI needle is used [48]. Recently, the utility of the sheathless porous tip interface was examined for the profiling of anionic metabolites in biological samples [49•], using exactly the same separation conditions as used for the profiling of cationic metabolites, only the MS detection and CE separation voltage polarity were switched/reversed. A broad range of anionic metabolite classes could be profiled under these conditions, including sugar phosphates, nucleotides and organic acids, as shown in Figure 3. An injection volume of circa 20 nL resulted in nanomolar detection limits, which corresponded to a significant enhancement as compared to the micromolar detection limits typically obtained with classical sheath–liquid CE–MS methods. Structural isomers of phosphorylated sugars as well as isobaric metabolites could be selectively analyzed by the proposed sheathless CE–MS method without using any derivatization. A front-end (partially) separation of these compounds is key in order to allow selective detection by MS. The methodology was applied to anionic metabolic profiling of glioblastoma cell line extracts. The low-pH separation buffer used for anionic metabolic profiling may not be the most optimal for achieving a baseline separation of structurally related sugar phosphates. Also important is that only anionic metabolites can be analyzed which are (partially) negatively charged under the used separation conditions. Still, the proposed single sheathless CE–MS approach can be used for the analysis of a wide range of highly polar anionic and cationic metabolites, thereby showing potential for global metabolic profiling studies.

Multi-segment injection for enhancing sample throughput

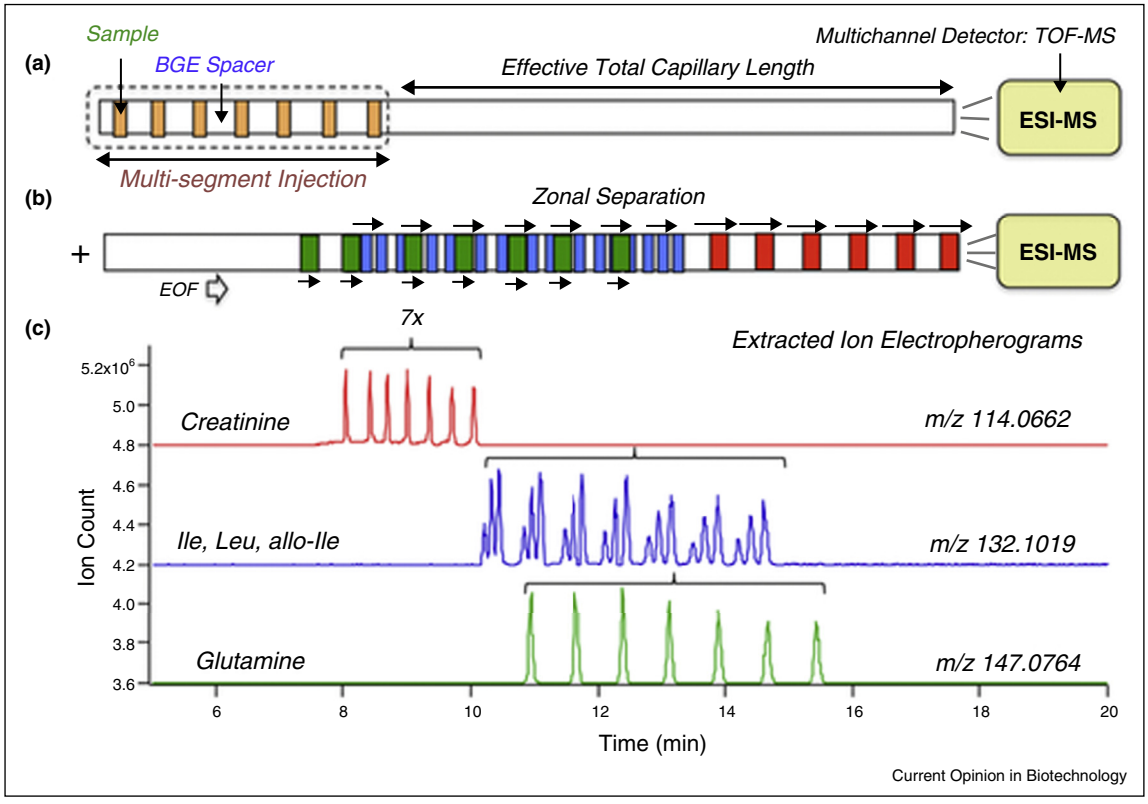
Clinical metabolomics studies require high-throughput analytical technologies. Currently, the flexibility of using shorter capillary lengths with the commercially available sheathless porous tip interface emitters is limited, that is, the dimensions of the porous tip capillary emitter are fixed (length is 90 cm and inner diameter is 30 μm). In this context, the multi-segment injection approach developed for CE–MS-based metabolomics studies by Kuehnbaum *et al.* may be used in sheathless CE–MS

Figure 3



Performance of CE–MS using a sheathless porous tip sprayer for anionic metabolic profiling. Multiple extracted ion electropherograms for the metabolite test mixture (25 μM) obtained with sheathless CE–MS in negative ion mode using a porous tip sprayer. Peaks: 1, 2-naphtol-3,6-disulfonic acid; 2, D(+)2-phosphoglyceric acid; 3, D-ribose-5-phosphate; 4, D-glucose-1-phosphate; 5, D-glucose-6-phosphate; 6, D-fructose-6-phosphate; 7, inosine 5′-monophosphate (IMP); 8, guanosine 3′,5′-cyclic monophosphate (cGMP); 9, guanosine 5′-monophosphate; 10, citric acid; 11, trimesic acid; 12, isocitric acid; 13, gluconic acid; 14, adenosine 3′,5′-cyclic monophosphate (cAMP); 15, 2-hydroxybutyric acid; 16, b-diphosphopyridine nucleotide (NAD+); 17, 3-hydroxybutyric acid. Experimental conditions: separation buffer, 10% acetic acid (pH 2.2); separation voltage, −30 kV (+0.5 psi applied at the inlet of the CE capillary); sample injection, 2.0 psi for 60 s.
Source: Reproduced from [49•] with permission.

Figure 4



Multi-segment injection in CE–MS. **(a)** Multiplexed separation based on serial injection of seven discrete sample segments within a single capillary by multi-segment injection CE–MS; **(b)** ions migrate as a series of zones in free solution before ionization; **(c)** the procedure enables reliable quantification of polar metabolites and their isomers in different samples as ionization occurs within a short-time interval (≈2–6 min) under steady-state conditions when using ESI–MS.
Source: Reproduced from [50••] with permission.

to enable high-throughput metabolic profiling of clinical samples [50••]. Multi-segment injection can be considered a multiplexing technique in which multiple samples are injected into the capillary before applying the separation voltage. Each sample is injected followed by the injection of a short plug of separation buffer which provides a differentiating gap between samples during the injection process. Careful optimization of the multi-segment injection process is critical in order to minimize overlap of the same metabolite peak from different sample injections during the electrophoretic separation. For example, the injection of too short separation buffer plugs between sample plugs may result in loss in resolution of metabolites and their isomers between adjacent sample plugs. The use of multi-segment injection in sheath–liquid CE–MS increased sample throughput up to one order of magnitude, thereby maintaining the separation of structurally similar metabolites without ion suppression (Figure 4). Overall, an acceptable precision was obtained for the quantification of various cationic metabolites in human plasma filtrates (RSD ≈ 10%, n = 70).

Concluding remarks

New CE–MS approaches have been developed for metabolomics showing improved analytical performances as compared to conventional CE–MS methods over the past few years. Here, the potential of CE–MS using a sheathless porous tip sprayer for the analysis of highly polar and charged metabolites has been highlighted. Though exquisite concentration sensitivities can be obtained with this approach, the next important step is to show its utility for large-scale clinical metabolomics studies. Such data are crucial to endorse the sheathless CE–MS method as a potential diagnostic tool. Sheathless CE–MS provides low nanomolar detection limits for a wide range of polar metabolite classes by only using an injection volume of 20 nL (or less) from a few microliters of sample in the vial. Therefore, this approach can be considered a highly attractive and unique analytical tool for probing the polar metabolome in ultra-small biological samples.

The use of an effective interfacing technique for CE–MS should preferably be used in conjunction with an effective injection strategy which allows the selective transfer

of target analytes into the CE system. Pre-analytics and injection are especially important for metabolic profiling of low-abundance metabolites in ultra-small biological samples. An efficient and selective transfer of ions into the separation capillary will significantly increase the durability of a single sheathless porous tip emitter, which at this stage can only be used for the analysis of up to 200 samples, and which may increase the overall sensitivity even further if a larger portion of the sample is injected. Further developments in this field will result in a viable CE–MS approach for probing the polar metabolome.

Conflict of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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- •• of outstanding interest

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